CE Solutions #2: Method development in CE: selecting your background electrolyte

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CE Solutions

separation | science
new horizons in chromatography and detection techniques

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Tech Tip

Method Development in CE: Selecting your background electrolyte

The selection of the proper background electrolyte (BGE) is key to a successful CE method. In this second issue of our series on CE Solutions we first look into the choices for BGE based on our analyte properties. Capillary electrophoresis (CE) is based on migration of charged components in an electric field. So when we start our method development, the first thing we need to consider is how to get our analytes to carry charge. We will look into the effects of pH on analyte charge and discuss opportunities to induce charge on neutral analytes. Additionally, we look into those aspects of a BGE that make the peaks sharp and the precision and robustness good.

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Method Development in CE:
Selecting your background electrolyte

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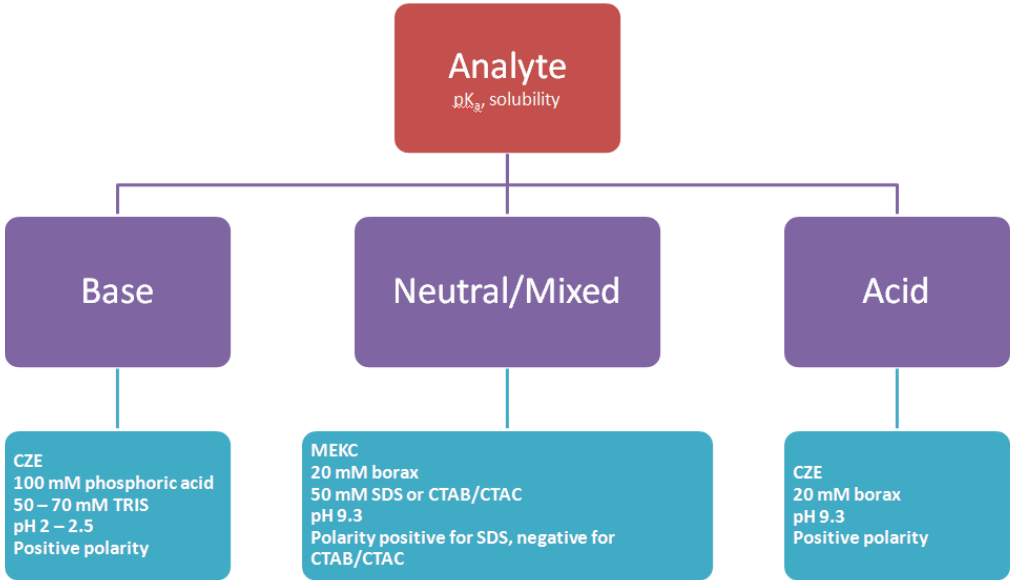
Charging through (de-) protonation

From the molecular structure and the active groups, we know or can estimate our analyte's pK_a(s). For an acid, if you have a solution above its pK_a, it is deprotonated and negatively charged. For a base, if the pH is below the pK_a, the base gets protonated and the compound will carry positive charge. Generally, we say that if you are at least two pH units away from the pK_a, the compound is completely protonated/deprotonated. If an analyte has multiple functional groups, such as a zwitterion, it carries a net charge at a certain pH that results from the sum of the individual charges of the functional groups.
So the first consideration for our separation medium, our background electrolyte BGE, is to select a pH at which our analytes are charged.

Induction of charge through interaction with BGE

Sometimes we have to deal with uncharged analytes, or a complex mixture for which there is no pH at which all analytes carry charge. We saw in the first issue of *CE solutions* that we need charge in order to separate in CE. So can uncharged analytes not be separated then? Not as such, but of course clever people found elegant solutions to that. One of these people is professor Terabe, who introduced MEKC, micellar electrokinetic chromatography, with micelles as a pseudo-stationary



phase to induce charge to otherwise uncharged components (see sidebar). Other people have developed on on that theme and published solutions such as micro-emulsion electrokinetic chromatography MEEKC, or charged cyclodextrins. Cyclodextrins are used for chiral separations, of which more in a later issue, but can also be beneficial to improve separation of achiral components. The common factor in all these applications is that there is a dynamic equilibrium of the (uncharged) analyte with a charged BGE component, thus inducing charge by way of a dynamic interaction. The net mobility for the analyte then depends on how strong the affinity for the charged BGE component is.

Why we need a buffering BGE

In order to fix the pH of the background electrolyte, it is not sufficient to adjust the pH of an arbitrary solution. As soon as we apply the voltage over our capillary, electrolysis of water occurs at the platinum electrodes. This means that at the cathode, water reacts with electrons into hydrogen gas and OH^-. At the anode, water reacts into oxygen and protons:

$$2\,H_2O \rightarrow O_2\,(g) + 4\,H^+ + 4\,e^-$$

$$2\,H_2O + 2\,e^- \rightarrow H_2\,(g) + 2\,OH^-$$

So as soon as we switch on the power supply, the pH in our inlet and outlet vials will change if we do nothing to prevent this. That is the reason that a BGE should always be a buffer. A buffer is a solution that is able to resist the pH shift that would otherwise be caused by a substantial addition of a strong acid or base. One of the few exceptions for using a buffering BGE is CE-MS, where the need for volatile BGE compounds overrules the need for buffering the BGE.

There are many buffers known in literature, so what is a good choice? Well, it depends (you will hear this more often). Inorganic buffers such as phosphate and borate are nice because they show very little UV absorption, which means that we can go to very low wavelengths such as 190–210 nm for sensitive detection, if needed. And they are cheap. But because they are small ions, often with multiple charges, they can result in relatively high currents. Zwitterionic buffers have the advantage that they have a low mobility, resulting in far lower currents than inorganic buffers. This means that higher concentrations and therefore higher buffering capacities can be used. The disadvantage is that these buffers absorb more in the low-UV region. My personal preference is to start simple, if there are no indications to do otherwise. That is, a phosphate buffer around pH 2–3 for basic analytes, and a borate buffer pH 9 – 9.5 for acidic analytes. Other commonly used buffers are listed in the table.

Table 1

Generic BGE	Composition
Phosphate pH 2.15 – 3.0	0.10 M H_3PO_4 with 0.05 – 0.09 M TRIS
Phosphate (reversed EOF dynamic coating) pH 2.15 – 3.0	0.10 M H_3PO_4 with 0.05 – 0.09 M triethanol-amine
Borate pH 9.3	20 mM sodium tetraborate
MEKC pH 9.3	20 mM sodium tetraborate with 50 mM sodium dodecylsulphate (SDS) or 10 mM cetyltrimethylammonium bromide (CTAB) or chloride (CTAC)
MEEKC	0.81% w/w octane, 6.61% w/w butan-1-ol, 3.31% w/w SDS, 89.27% w/w 10 mM sodium tetraborate pH 9.2 E.g. *in K.D. Altria, J. Chromatogr. A 844 (1999) 371-386*

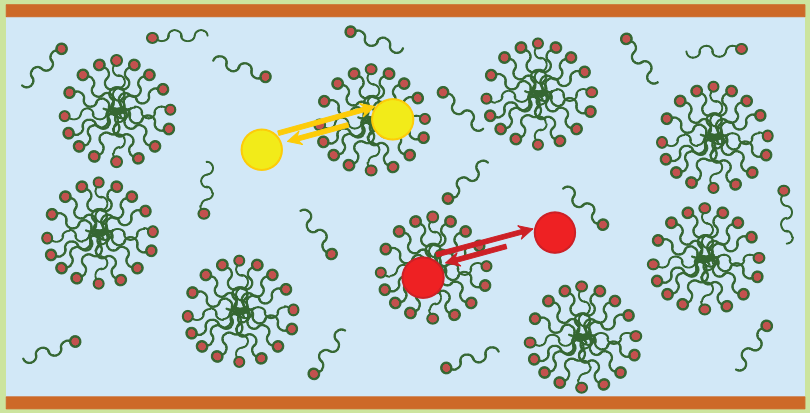
Table 2

Common buffers	pK_a
Phosphoric acid	2.15
Malonic acid	2.85
Formic acid	3.75
6-Aminocaproic acid	4.37
Acetic acid	4.76
MES	6.21
Triethanolamine	7.76
TRIS	8.06
Tricine	8.26
Borate	9.23

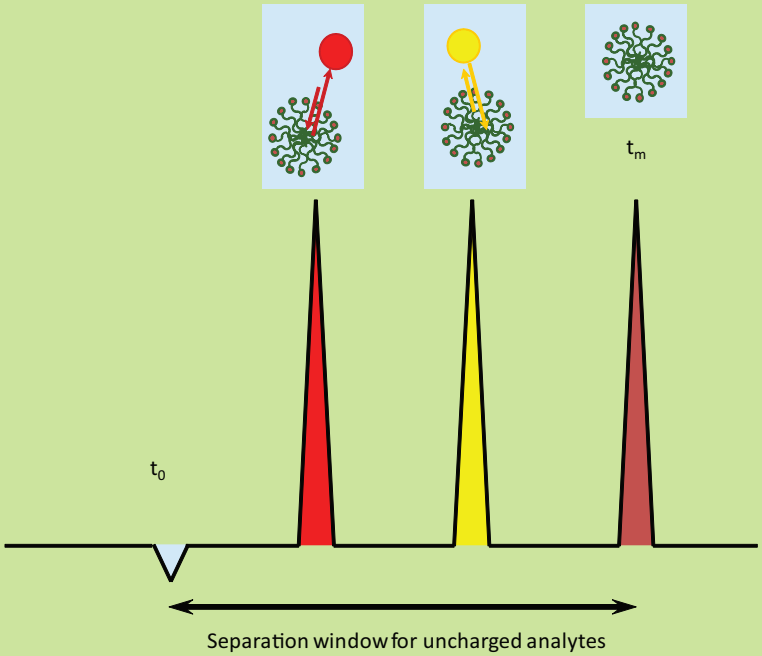
Buffering capacity and current

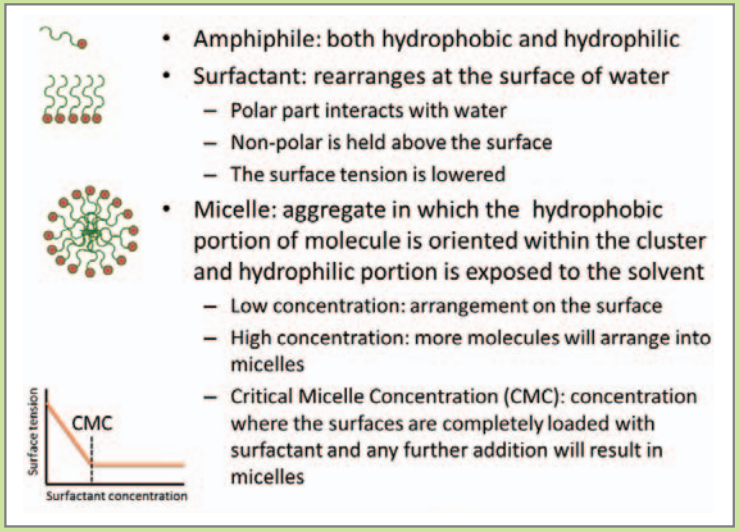
In order to have a good buffering capacity, we would like to have as high a buffer concentration as possible. But the higher the buffer concentration, the higher the current. At a certain point, the heat from the current can no longer be dissipated from the capillary. Excessive Joule heating will cause band broadening, resulting in broader peaks and reduced resolution. An Ohm's plot (see sidebar) can tell us when this heating becomes problematic. To

Micellar Electrokinetic Chromatography (MEKC)



Prof. Terabe introduced micellar BGEs in order to separate uncharged components with the capillary electrophoresis techniques (S. Terabe et al. *Anal. Chem*. 56 (1984) 111). If we dissolve a surfactant, an amphiphilic molecule with a hydrophilic and a hydrophobic end, in an aqueous solution, it will initially rearrange at the surface. The polar part interacts with the water, the apolar part with the air. This will reduce the surface tension, hence the name surfactant. If the concentration of the surfactant increases, a limit is passed at which the surfactant starts forming aggregates in which the hydrophobic portion of molecule is oriented within the cluster and hydrophilic portion is exposed to the solvent. This is called a micelle and the limit the critical micelle concentration (CMC). The most commonly used surfactant in MEKC is sodium dodecyl sulphate, SDS.



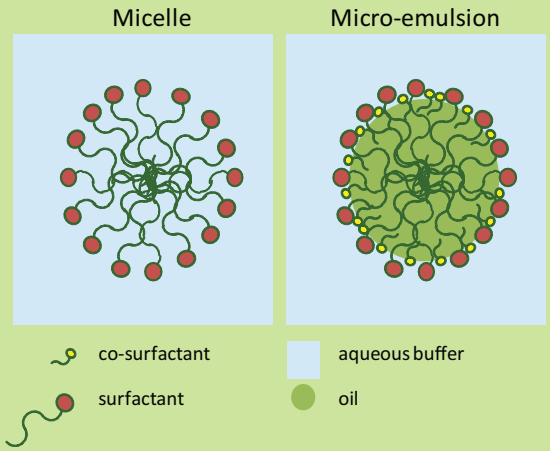


The micelle has a hydrophobic inner and hydrophilic outside. The micelles carry charge on the outside and, therefore migrate differently from the EOF. Analytes can partition in these micelles, a form of chromatography. Lipophilic analytes (yellow analyte in the illustration) will prefer the hydrophobic inner, while hydrophilic analytes (red) will prefer the aqueous phase. If a neutral analyte interacts strongly with the micelles, the net resulting mobility will be close to the mobility of the micelles. If a neutral analyte interacts little with the micelle, the net mobility will be close to the EOF. Hence the migration time window for these neutral compounds is between the EOF and the migration of the micelles.The result is a separation of the neutral analytes based on their affinity for the micelles.

For charged analytes, the net mobility results from a combination of the electrophoretic mobility and the interactions with the micelle. The latter can be both electrostatic interactions with the charged outside and hydrophobic interactions with the inner of the micelle.

Micro-emulsion electrokinetic chromatography (MEEKC)

In MEEKC, a micro-emulsion is used instead of a micellar solution. A micelle consists only of surfactant molecules oriented in spheres with the polar groups on the outside. A micro-emulsion consists of oil drops surrounded by surfactant and co-surfactant molecules. A common micro-emulsion is made of borate buffer (aqueous phase), SDS or CTAB (surfactant), butanol (co-surfactant) and octanol (oil). The use of a micro-emulsion instead of a micelle generally benefits the separation of analytes with relatively high lipophilicity.



prevent it, we can either reduce the voltage, the capillary diameter or the buffer conductivity. The latter is reduced by lowering the concentration or by choosing another type of buffer, like zwitterionic buffers.

Buffer co-ion and mobility matching

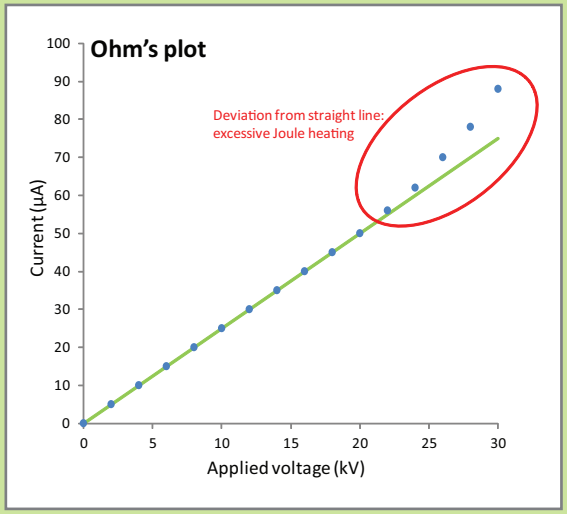
The kind of ion in our BGE-buffer that co-migrates with our analytes, that is, our buffer co-ion, can also be of importance. Peak shapes are most symmetric (least electromigration dispersion) if the migration of the buffer co-ion is close to the migration of our analytes. Finding a co-ion that does this, is sometimes referred to as mobility matching. For instance, if you are working with small molecule basic pharmaceutics, you will often find that it is beneficial for your peak shape to use TRIS-phosphate buffer instead of sodium phosphate buffer at low pH, as is illustrated in the side bar.

The effect of the BGE on precision

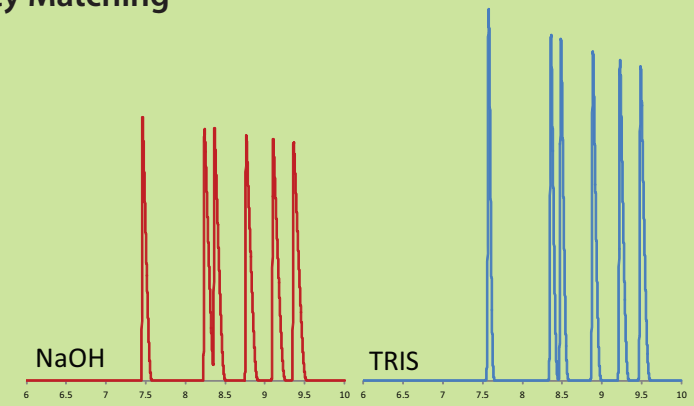
In order to improve precision in capillary electrophoresis, think about the significance of all the steps we do. Here we focus on the accurate description and preparation of the buffer. In literature, we find many different ways to make something as simple as a phosphate buffer, and in the end we often still don't know exactly how it was prepared or what the precise composition was. We saw in the previous issue that factors such as the pH and ionic strength influence the electro-osmotic flow. It is thus important to control the pH and ionic strength so that they are as similar as possible from preparation to preparation. This means in my opinion to avoid as much as possible producing buffers by titrating to the right pH. Instead, calculate what amounts are needed (there are many software packages, websites and books available on this topic) and

Current

Current is created by the movement of charges particles in an electric field. The current for a certain CE system depends on the capillary diameter, the field strength and the conductivity of the buffer. The latter is directly proportional to the ionic strength and mobility of the buffer components. The ionic strength not only depends on the buffer concentration, but also on the charge of the buffer ions. Mobility is size related. So multiple-charged, small buffer components give rise to relatively high currents in CE buffers. Zwitterionic buffers result in low currents. The best way to check whether a certain current is too high and results in excessive Joule heating in our system, is by making an Ohm's plot. An Ohm's plot is made by varying the applied voltage and measuring the resulting current. A plot of the results should be a straight line. If the current starts deviating from the straight line, more heat is generated than can be dissipated, which will result in band broadening. The combination of applied voltage, buffer and capillary diameter is unfavourable. To reduce the current and with that the excessive Joule heating for our system, we can either reduce the voltage, the capillary diameter or the buffer conductivity. The buffer conductivity is reduced by reducing the concentration or by choosing another type of buffer, like zwitterionic buffers.



Mobility Matching



Having a buffer co-ion with a mobility that matches better with the mobility of the analytes, electromigration dispersion can be reduced and peak shapes and resolutions are better. This example is from a series of basic compounds, in migration order: procaine, lidocaine, tetracaine, trimecaine, leucinocaine and bupivacaine. The BGE consists of 30 mM phosphoric acid with 20 mM of sodium hydroxide (red) or TRIS (blue), and has a pH of 2.6. TRIS has a better mobility match with the analytes, resulting in higher, sharper and better resolved peaks. The E-grams are calculated with PeakMaster 5.3, a freeware program from prof. Bohuslav Gaš and his Group of Electromigration Separation Processes at the Charles University of Prague.

create a precise recipe. For instance, 100 mmol/L phosphoric acid plus 70 mmol/L of a strong base, such as NaOH, TRIS or Triethanolamine, will result in a BGE with a pH of 2.5 and a defined composition and thus ionic strength.

"Borate buffer" is another of these unspecific ones. Better is to say for example 20 mmol/L sodium tetraborate, which has a pH of 9.3. Alternatively, you can purchase commercially available BGEs and kits that are especially designed for CE. Note that some buffers such as TRIS are rather temperature dependent. This is mostly expressed as dpK_a/d_T, the change in pK_a per degree. For example, for TRIS this value is -0.028/°C (*Buffer Solutions, RJ Beynon and JS Easterby, Oxford University Press, ISBN 0-19-963442-4*). So for every degree C increase in temperature, the pH of the TRIS buffer will decrease with 0.028 pH unit. If we prepare our buffer at 20 °C and run at 30 °C, this will give in the case of TRIS a pH change of e.g. from 8.1 to 7.8.

BGE and analyte pK_as and robustness

If we need to separate two compounds with similar molecular masses but slightly different pK_as (e.g., positional isomers), from an electrophoretically point of view, we would select a pH that is exactly in between the pK_as of the compounds. In that case, with similar masses and maximum charge difference, we obtain the largest charge over mass ratio difference in a CZE system. However, from a robustness point of view, this might not be our best option. A tiny change in pH will immediately result in charge changes on our compounds and, therefore, in mobility changes, reducing resolution and causing migration time shifts. We would need a very stringent control of the pH that might be over the practical range of the buffering capacity of our BGE. So, if a robust method is required for long-term QC type of use, we might be more secure by selecting a BGE with a pH more than 2 pH units away from the pK_as of our compounds so that the compounds are fully charged and minor pH-shifts will not influence their relative mobilities. If there is insufficient separation in that case, consider using micelles (MEKC system) or cyclodextrins to improve separation by way of dynamic interaction.

Wrapping up on background electrolyte

Selecting our BGE is more than picking a pH for our separation. We discussed opportunities to induce charge on uncharged analytes as well as the effect of the BGE composition on stacking, mobility matching, current and excessive Joule heating. In the previous issue we saw that the electro-osmotic flow is influenced by BGE parameters such as ionic strength and viscosity, and that between pH 4 and 7 a small change in pH results in a large change in EOF. We reviewed in this issue the importance of buffering BGEs, mobility matching and precise recipes for improved precision. Sometimes this is insufficient and additional measures are needed to control the EOF by controlling the charge of the capillary wall. In the next issue of *CE Solutions* we will look into that.

Cari Sänger has more than 20 years of experience in pharmaceutical and chemical analysis. Her aim is to stimulate people to keep growing and learning, striving to get the best out of themselves. Cari is an independent, reliable, scientific people-manager and a globally recognized expert on separation science, especially within the capillary electrophoretic techniques. Cari's focus is primarily on implementation, knowledge transfer and good working practices.

FEATURED WORKSHOP

CE Method Development
25 April, 2012 - Basel, Switzerland
Price: US$795

Who should take this course?
Analytical scientists and technicians who want a better understanding of the capillary electrophoresis techniques and practical tips for method development. This course is designed for analysts who use CE as a part of their regular jobs, but technicians with some CE experience will also find the course valuable. No previous CE training is assumed, however, much of the course will appeal to the experienced method developer who wants to stand on firm ground in the basics of CE. Also lab managers who need to supervise CE method development and review CE methods will benefit.

Click here to learn more>>

CE Method Validation
26 April, 2012 - Basel, Switzerland
Price: US$795

Who should take this course?
Analytical scientists and technicians who are responsible for validating capillary electrophoresis methods. The course is also useful for managers and QA staff involved in the method validation process. For workers who develop, but do not validate methods, this course will give insight into how to develop methods that will be easier to validate. No prior CE experience is needed, although those with practical CE experience and those who attended the CE method development course will certainly benefit more than those with no experience at all.

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Ask the Doctor

Cari Sänger is available to answer your specific method development and troubleshooting CE questions. Submitted Q & As will also form the basis of future CE Solutions.

NOTE! *"Help! I need a method to separate ____" Unfortunately, this is a question that Cari can't help you with. However, here are a few hints: (1) do a literature search using 'Pub Med' or one of the free search engines; (2) a good source of methods are Electrophoresis Journal of Chromatography A and B issues; (3) consult the applications literature of various manufacturers (4) visit Chrom Forum at www.chromforum.org*

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